IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Tuszynski, Mark H.

Title: METHODS FOR THERAPY OF NEURODEGENERATIVE DISEASE OF THE BRAIN

Appl. No.: 09/620,174

Filing Date: 07/19/2000

Examiner: Chen, Shin-Lin

Art Unit: 1632

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231, on the date below.

Michelle Sympson
(Printed Name)

Michelle Sympson
(Signature)

April 7, 2003
(Date of Deposit)

DECLARATION OF MARK H. TUSZYNSKI, M.D.

I, Dr. Mark H. Tuszynski, declare as follows:

1. I am a Professor of Neurosciences and Director of the Center for Neural Repair at the University of California, San Diego (UCSD). I practice medicine as an attending neurologist at the UCSD and VA Medical Centers in La Jolla, California, and as the lead neurologist in ongoing clinical trials for treatment of Alzheimer's Disease (AD) using gene therapy in humans. I am also an inventor of the invention claimed in this US Patent Application Serial No. 09/060,543 (methods for *in vivo* gene therapy of neurodegenerative conditions), and in U.S. Patent No. 6,167,888 (claiming methods for *ex vivo* gene therapy of neurodegenerative conditions).

Background

2. The *in vivo* gene therapy method which is the subject of the present patent application has been tested in non-human primates and rodents, in preparation for human clinical studies to begin later this year. The *ex vivo* method which is the subject of the '888 patent is already being used in clinical trials, for treatment of AD.

3. Effective gene therapy generally requires that a therapeutic gene product be expressed in target cells at levels, and for a duration, sufficient to provide a therapeutic benefit, without harmful immune responses to the delivery vehicle or gene product. In gene therapy of neurodegenerative disease, the goal is not gene replacement, nor is it permanently active expression of a therapeutic neurotrophin in the brain. Instead, relatively transient expression of neurotrophins can be sufficient to stimulate neuronal growth to a therapeutically significant degree. Thus, the eventual loss of expression (whether through immune processes, loss of promoter activity or other causes) is of less concern in the invention than in other therapeutic contexts.

4. Further, in the relatively immune-protected environment of the brain, immune responses to exogenous material are typically muted. In this respect, our findings are consistent with recent evidence indicating that viral vectors elicit only transient inflammation, if any, when *inserted directly* into brain parenchyma (as is done in the invention), versus entry by other means of delivery into the brain (e.g., infusion).

Overview of Data

5. We have used adeno-associated viral vectors to deliver neurotrophins by *in vivo* and *ex vivo* methods into the brains of humans, and have tested lentiviral vectors to the same ends in non-human primates. With particular respect to the *in vivo* work that is the subject of this patent application, we utilized aged monkeys which model neurodegeneration experienced in Parkinson's Disease (PD) or Alzheimer's Disease (AD). Animals modeling PD were treated with lenti-glial derived neurotrophic factor (GDNF), with lenti-ß-galactosidase (GAL) serving as a control. Animals modeling AD were treated with lenti-nerve growth factor (NGF), with lenti-enhanced green fluorescent protein (eGFP) serving as a control.

6. In these experiments, we achieved expression of neurotrophin in targeted neurons at a level, and for a duration, believed to be sufficient for our therapeutic goals. Indeed, as many as >90% of targeted neurons can be efficiently transduced using the method of the

invention, a visible demonstration of which is provided in enclosed Declaration Figure 1 (Dec.Fig. 1). In the Figure, GFP labeled treated neurons (using antibodies to glial filament protein (GFAP) or NeuN neuronal nuclear protein) show bright green if infected by the vectors employed in our experiments (a few such neurons are highlighted with arrows in each of the three photographs included in Dec. Fig. 1). Further, neither significant anti-vector immune responses nor cytotoxicity have been experienced in treated animals or people.

7. Representative data in these respects (not including confidential data from the human clinical trials) is provided in the present patent application, at page 7 and page 20 (Example II); in co-pending U.S. patent application 10/032,952; and in this Declaration.

PD Animal Models: Protocol, Expression and Lack of Immune Response

8. In the PD animal models, treatment with GDNF according to the invention stimulated increased production of dopamine in the brain (a critical protein for mitigation of PD), increased neuronal density around treated areas and, most significantly, resulted in improved motor function as measured by several criteria in treated animals.

9. More specifically, each monkey received six stereotaxic injections of lenti- Gal or lenti-GDNF bilaterally into the caudate nucleus, putamen, and substantia nigra. Injections were made into the head of the caudate nucleus (10 µl), body of the caudate nucleus (5 µl), anterior putamen (10 µl), commissural putamen (10 µl), postcommissural putamen (5 µl), and substantia nigra (5 µl). Injections were made through a 10-µl Hamilton syringe connected to a pump at a rate of 0.5 µl/min.

10. The left side was injected 6 weeks before the right. During the first surgical session, there was a technical failure with the virus aggregating in the needle, which prevented its injection into the brain. Thus, the left side served as an additional control for the right side. Postmortem, all GDNF injections were localized to the caudate nucleus, putamen, and supranigral regions, as revealed by standard staining procedures.

11. Expression levels of the neurotrophin were confirmed by *in vitro* measurement of immunoreactivity with antibody in post-mortem tissue sections. All aged monkeys receiving lenti-GDNF displayed robust GDNF immunoreactivity within the right striatum (Dec.Fig. 2A) and substantia nigra (Dec.Fig. 2C), indicating expression and secretion of the neurotrophin had occurred. No monkeys receiving lenti-ß-Gal displayed specific GDNF immunoreactivity in the right striatum (Dec.Fig. 2B), but each did display robust expression of ß-Gal. No immune staining for antibodies against the lentiviral vector itself was observed.

12. Sections from all monkeys were stained for CD45, CD3, and CD8 markers to assess the immune response after lentiviral vector injection. These antibodies are markers for activated microglia, T cells, and leukocytes including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes. Staining for these immune markers was weak, and often absent, in these animals. Mild staining for CD45 and CD8 was seen in two animals. Other monkeys displayed virtually no immunoreactivity even in sections containing needle tracts.

13. Expression of GDNF in treated animals persisted for a relatively long duration, sufficient to stimulate the neuronal growth sought in the invention. GDNF-containing fibers emanating from putaminal injection sites were seen coursing medially toward and into the globus pallidus (Dec.Fig. 2D). These staining patterns were clearly distinct from the injection site and respected the boundaries of the striatal target structures. In contrast, anterograde transport of ß-Gal was not observed in lenti- Gal monkeys. This suggests that secreted GDNF, and not the virus per se, was anterogradely transported.

Neurotrophin-induced improvements in motor function and neuronal density in PD animal models

14. In response to the therapy, dopamine levels in GDNF treated animals increased significantly. Aged monkeys underwent fluorodopa (FD) positron emission tomography (PET) before surgery and again just before being killed. Before treatment, all monkeys displayed symmetrical FD uptake in the caudate and putamen bilaterally (ratio: 1.02 ± 0.02). Three of four lenti-GDNF-treated monkeys displayed clear increases in FD uptake on the treated side. Within the striatum, lentiviral delivery of GDNF increased a number of markers of dopaminergic function. For example, relative to control animals, measurement of dopamine (DA) and homovanillic acid (HVA) revealed significant increases in the right caudate nucleus (140% DA, $P < 0.001$; 207% HVA, $P < 0.001$) and putamen (47.2% DA, $P < 0.05$; 128% HVA, $P < 0.01$) in lenti-GDNF-treated aged monkeys.

15. Neuronal density (growth) in treated animals also increased significantly. Stereological counts revealed an 85% increase in the number of TH-immunoreactive nigral neurons on the side receiving lentivirally delivered GDNF relative to control animals. Further, a 35% increase in neuronal volume was seen on the GDNF-treated side of the brain in lenti-GDNF-injected aged monkeys (lenti- Gal 10,707.5 ± 333 μm3; lenti-GDNF 16,653.7 ± 1240 μm3; $P < 0.001$).

16. Perhaps most significantly, an improvement in function was observed on treatment of animals using a second model of neurodegenerative changes similar to those occurring in PD. In these experiments, young adult monkeys received unilateral intracarotid injections of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to induce extensive nigrostriatal degeneration, resulting in a behavioral syndrome characterized by robust motor deficits.

17. 20 young adult rhesus were initially trained 3 days per week until asymptotic performance was achieved on a hand-reach task in which the time to pick up food treats out of recessed wells was measured. Each experimental day, monkeys received 10 trials per hand. Once per week, monkeys were also evaluated on a modified parkinsonian clinical rating scale (CRS). All monkeys then received an injection of 3 mg MPTP-HCl into the right carotid artery, initiating a parkinsonian state.

18. One week later, monkeys were evaluated on the CRS. Only monkeys displaying severe hemiparkinsonism with the classic crooked arm posture and dragging leg on the left side continued in the study (n = 10). Monkeys with this behavioral phenotype generally display the most severe lesions neuroanatomically and do not display spontaneous recovery behaviorally.

19. On the basis of CRS scores, monkeys were matched into two groups of five monkeys, which received on that day lenti-ß-Gal or lenti-GDNF treatment. Using magnetic resonance imaging (MRI) guidance, all monkeys were given lentivirus injections into the caudate nucleus (n = 2), putamen (n = 3), and substantia nigra (n = 1) on the right side using the same injection parameters as in experiment 1. One week later, monkeys began retesting on the hand-reach task three times per week for 3 weeks per month.

20. For statistical analyses, the times for an individual week were combined into a single score. During the weeks of hand-reach testing, monkeys were also scored once per week on the CRS. Individuals blinded to the experimental treatment performed all behavioral assessments. Statistically significant differences between lenti-GDNF and lenti-ß-Gal were discerned at post-treatment observations 6, 7, 8, and 9 (Kolmogorov-Smirnov test, $P < 0.04$ for each comparison).

21. Lenti-GDNF-treated animals also improved performance on the operant hand-reach task. Under the conditions before MPTP administration, animals in both groups performed this task with similar speed. However, after MPTP, all lenti-ß-Gal-treated

animals were severely impaired, with monkeys often not performing at all, or requiring more than the maximally allowed 30 s. In contrast, three of the four lenti-GDNF monkeys performed the task with the left hand at near-normal levels, whereas one lenti-GDNF-treated monkey was impaired and performed this task in a manner similar to the lenti-ß-Gal-treated animals.

AD Animal Models: Protocol, Expression and Lack of Immune Response

22. In the AD animal models, treatment with NGF according to the invention increased neuronal density around treated areas and, most significantly, resulted in improved cognitive function as measured by several criteria in treated animals.

23. An animal model that mimics loss of cholinergic neurons in AD is transection of the fornix pathway connecting the septum from the hippocampus. Such transections cause retrograde degeneration of cholinergic and non-cholinergic cell bodies in the septal nucleus of rats and primates. 7 rats underwent fornix transaction, and an equal number of animals were utilized as controls.

24. Treatment protocols were followed, and responses to treatment evaluated, as described above with respect to the GDNF treated animals, except that treatment focused on the Ch4 region of the forebrain, a principal region of impairment in AD. Two injection sites were chosen per hemisphere. The vector dosage concentration (both of NGF expressing vectors for treatment, and GFP expressing vectors for controls) applied was 1.5 µl/site, delivered over an interval of 5 minutes. Animals were sacrificed, and tissue analyzed, 3 weeks after treatment.

Neurotrophin-induced improvements in motor function and neuronal density in AD animal models

25. As visually apparent from Dec.Fig. 1, neurons in the treated regions displayed vigorous GFP immunoreactivity in histology, indicating extensive transfection of target cells by the NGF expressing vectors. Neither cytotoxicity nor an immune response to the vector

constructs (using CD3 and CD8 as markers) was observed (CD3: Dec.Fig. 3A-C; and CD8: Dec.Fig.3D).

26. As shown in Dec.Figs. 4-6, neuronal density (as measured by number [Dec.Figs. 4 and 6], and size [Dec. Figs. 5 and 6] of neurons) in treated animals increased significantly, approaching near normal levels (compare, e.g., Dec.Fig. 6C and B showing density measured in, respectively, young and NGF treated animals, versus aged control animals [Dec.Fig. 6A]).

27. Moreover, the growth was associated with increased expression of the p75 receptor. Expression of p75 is regulated by NGF, so that a loss of NGF signalling further reduces the amount of p75 present in the brain, which may contribute to a decline in retrograde NGF signalling.

28. There were significantly fewer p75-labeled neurons in Ch4 from untreated aged rats than in untreated young rats ($p<0.01$). However, on treatment of the aged animals, the mean number of p75-labeled Ch4i neurons from NGF-grafted aged rats increased to a level not significantly different from numbers observed in the untreated young animals. See, Dec.Fig. 4.

29. The increases in neuronal density observed were of cholinergic neurons--those lost in AD. See, Dec.Fig. 7. These results demonstrate that cholinergic neurons in the rat brain can be rescued from loss through intraparenchymal delivery of NGF using the method of this invention.

Conclusion

30. From all of the foregoing results, as well as those outlined in the patent application, one can reasonably predict that the method of the invention may be successfully and effectively practiced in humans, to produce a therapeutic response to neurotrophins delivered for expression from vector constructs.

31. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements are so made punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

April 7, 2003
Dated

Dr. Mark H. Tuszynski